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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Rasmussen et al.

Serial Number: 10/814,025

Filing Date: March 31, 2004

Examiner: David M. Sullivan

Group No.: 1636

Title: GLUCOCEREBROSIDASE HAVING EXPOSED
MANNOSE RESIDUES

Commissioner for Patents
P.O. Box 1450
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. 1.132

I, Timothy Edmunds, hereby state and declare in writing as follows:

1. I am Vice President, Therapeutic Protein Research, at Genzyme Corporation ("Genzyme"). My Curriculum Vitae is attached hereto as Exhibit A.
2. Since, 1989, I have been personally involved in Genzyme's research and development programs relating to glucocerebrosidase and the commercial products Ceredase® and Cerezyme®.
3. In my current position, I am responsible for directing Genzyme's Lysosomal Storage Disease (LSD) research efforts as well as the Protein Characterization, Protein Engineering and Mass Spectrometry groups. In such capacity, I have responsibility for structural and functional characterization of Genzyme's therapeutic protein products.

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4. I have reviewed the specification and currently amended claims of U.S. Serial Number 10/841,025 ("the '025 application") as well as the Office Actions and responses thereto filed or received by applicants in connection with the prosecution thereof. I make this Declaration to address certain of the issues raised by the Examiner in the Office Action mailed January 19, 2006 and in the Interview Summary mailed March 15, 2006. Specifically, I make this Declaration to address the Examiner's basis for rejecting the pending claims of the '025 application as failing to comply with the written description and enablement requirements of 35 U.S.C. § 112.

5. Regarding the written description requirement, I understand that the Examiner believes that the term "human glucocerebrosidase" as recited in the claims encompasses a genus of numerous different enzymes which cause hydrolysis of a glucocerebroside, and that the specification does not disclose a representative number of species within that genus. I further understand that the Examiner is of the view that there is no evidence to support applicants' assertions that treatment of cultured mammalian cells with inhibitors of carbohydrate processing that act to inhibit the conversion of $\text{Glc}_3\text{Man}_9\text{GlcNac}_2$ to smaller species leads to production of glucocerebrosidase with exposed mannose residues, which glucocerebrosidase molecules would have a higher affinity for the human mannose receptor.

6. Regarding the enablement requirement, I understand that the Examiner believes that the skilled artisan would not be able to make and use the claimed invention because, according to the Examiner, the claims encompass "any polypeptide capable of hydrolyzing a glucocerebroside isolated from any mammalian cell wherein conversion of $\text{Glc}_3\text{Man}_9\text{GlcNac}_2$ to smaller species has been inhibited." Specifically, the Examiner is apparently of the view that because the features that define the genus of such polypeptides purportedly are not adequately disclosed, and because of the unpredictability of glycosylation in mammalian cells, "the skilled artisan would have to make and test each species within an essentially unlimited genus of polypeptides for the function recited in the claims."

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7. In sum, and as summarized in the Interview Summary, I understand the Examiner is of the view that the "actual production of a glucocerebrosidase suitable for therapeutic use would be unpredictably dependent upon the mammalian cell and the inhibitor of carbohydrate processing used to produce the glucocerebrosidase."

8. At the outset, I note that there is currently believed to be only one human glucocerebrosidase. The sequence of the human glucocerebrosidase gene, and the amino acid sequence it encodes, is well known in the art (EC 3.2.1.45). Therefore, one of ordinary skill in the art would not understand the term "human glucocerebrosidase" as recited in the claims to refer to a "genus" of proteins. Rather, one of ordinary skill in the art would understand it to refer to a single human protein of known amino acid sequence.

9. To address the Examiner's concerns on written description and enablement, I have supervised experiments that demonstrate that a skilled artisan, following the teachings of the '025 application, can control the glycosylation process in mammalian cell culture to produce glucocerebrosidase containing a higher number of exposed mannose residues than glucocerebrosidase recovered from untreated cells. Specifically, the '025 application teaches that one would do this by treating a culture of mammalian cells capable of expressing glucocerebrosidase with an inhibitor of carbohydrate processing that acts to inhibit the conversion of $\text{Glc}_3\text{Man}_9\text{GlcNac}_2$ to smaller species. Following these teachings, and using inhibitors of carbohydrate processing available as of the priority date, we have treated cultures of mammalian cells capable of expressing human glucocerebrosidase with a variety of such inhibitors and shown that all are useful to produce glucocerebrosidase with a higher number of exposed mannose residues than glucocerebrosidase recovered from untreated cells.

10. The first experiment we conducted to demonstrate this is detailed in Exhibit B. In this experiment, cultures of Chinese hamster ovary (CHO) cells stably transfected with a gene encoding glucocerebrosidase were treated with four different inhibitors of carbohydrate processing encompassing three different classes: castanospermine and deoxynojirimycin (both glucosidase inhibitors);

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deoxymannojirimycin (a mannosidase I inhibitor); and swainsonine (a mannosidase II inhibitor). In order to analyze the sugar structures produced as a result of such culture, we purified the glucocerebrosidase from the culture medium and treated the samples overnight with Endoglycosidase H ("Endo H") under standard conditions. Endo H is an enzyme that removes oligomannose type and hybrid type sugar structures but would have no effect on glucocerebrosidase containing complex sugars. In order to determine if the glucocerebrosidase samples contained exposed mannose and were therefore sensitive to Endo H, we ran the samples on 4-12% SDS PAGE gel and stained the gel with Coomassie blue.

11. The results are shown in Figure 1 of Exhibit B. Lane 1 of the gel contains protein molecular weight standard. Lanes 2-6 contain glucocerebrosidase without Endo H pretreatment; lanes 7-11 contain glucocerebrosidase with Endo H pretreatment. Lanes 2 and 7 contain glucocerebrosidase from cells cultured in the absence of any inhibitor of carbohydrate processing; lanes 3 and 8 contain glucocerebrosidase from cells cultured in the presence of deoxymannojirimycin; lanes 4 and 9 contain glucocerebrosidase from cells cultured in the presence of swainsonine; lanes 5 and 10 contain glucocerebrosidase from cells cultured in the presence of castanospermine; lanes 6 and 11 contain glucocerebrosidase from cells cultured in the presence of deoxynojirimycin. As can be seen in Figure 1, upon treatment with Endo H, there was no change in migration between lanes 2 and 7, which were glucocerebrosidase samples recovered from cells not exposed to inhibitors of carbohydrate processing. In contrast, all of the samples obtained from CHO cells treated with inhibitors of carbohydrate processing showed a migration shift upon treatment with Endo H, indicating the presence of an increased number of oligomannose residues and therefore exposed mannose residues.

12. The presence of exposed mannose residues on glucocerebrosidase recovered from cells treated with inhibitors of carbohydrate processing was further confirmed by mass spectrometry. Figure 2 of Exhibit B shows the MALDI-TOF MS (Matrix Assisted Laser Desorption Ionization - Time of Flight Mass Spectrometry) spectra of the oligomannose-containing glycans released from Endo H treatment of

glucocerebrosidase produced by culturing mammalian cells in the presence of the four different inhibitors of carbohydrate processing. As can be seen in Figure 2, while glucocerebrosidase recovered from cells cultured without an inhibitor (see control) contains little high mannose or hybrid type oligosaccharides (mainly Man5, Man5P and Man6P), cells cultured in the presence of deoxymannojirimycin produced glucocerebrosidase with Man9, Man8, Man7, Man6P, Man 6 and Man5 structures, cells treated with swainsonine produced glucocerebrosidase with hybrid type oligosaccharides and cells treated with castanospermine and deoxynojirimycin produced glucocerebrosidase with either Man9 or Man9/Man8 structures. Thus, treatment of CHO cells with any of four different inhibitors of carbohydrate processing that act to inhibit the conversion of $\text{Glc}_3\text{Man}_9\text{GlcNac}_2$ to smaller species all produce glucocerebrosidase containing a higher number of exposed mannose residues than would be contained on glucocerebrosidase recovered from untreated cells.¹

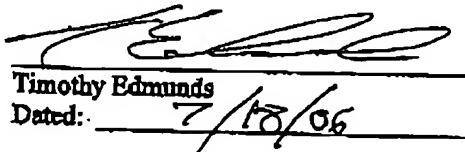
13. The second set of experiments, detailed in Exhibit C, demonstrate that the same results can be obtained with various different human cell lines. Figure 1 is a Western blot of glucocerebrosidase obtained from HeLa cells (a human cancer cell line) transduced with an adenoviral vector expressing glucocerebrosidase and cultured in the presence of each of the four inhibitors of carbohydrate processing. The glucocerebrosidase was either treated or not treated with Endo H, applied to a 4-12% SDS PAGE gel, transferred to a PVDF membrane and incubated with biotinylated rabbit anti-human glucocerebrosidase antibody. Similar to what was observed with the CHO cells (see Exhibit B, and discussion, *supra*), the samples from HeLa cell cultures treated with the inhibitors are sensitive to Endo H as evidenced by faster migration after Endo H treatment, although the effect was greater in cells treated with deoxymannojirimycin and swainsonine. Thus, it is clear that treatment of this human cell line expressing glucocerebrosidase with inhibitors of carbohydrate processing results in the production of glucocerebrosidase having more exposed mannose residues than glucocerebrosidase recovered from untreated cells.

¹ Note that in Figure 2, there is a scale difference in the control sample as indicated by the higher baseline noise.

14. We demonstrated a similar result with a different human cell line. Figure 2 of Exhibit C is a Western blot of glucocerebrosidase obtained from HEK 293 cells (a human kidney cell line) cultured in the presence of the four different inhibitors of carbohydrate processing. As can be seen in Figure 2, culture of HEK 293 cells in the presence of the inhibitors results in the production of glucocerebrosidase containing higher amounts of exposed mannose residues than glucocerebrosidase recovered from untreated cells.

15. In conclusion, the data set forth herein demonstrate that glucocerebrosidase produced in three different mammalian cell lines (CHO, HeLa and 293) treated with four different inhibitors of carbohydrate processing that act to inhibit the conversion of $\text{Glc}_3\text{Man}_9\text{GlcNac}_2$ to smaller species, contains a higher number of exposed mannose residues than glucocerebrosidase recovered from untreated cells. In my opinion, these data are sufficiently strong and complete that they are fairly extrapolated to other mammalian cell types and other inhibitors of carbohydrate processing that act to inhibit the conversion of $\text{Glc}_3\text{Man}_9\text{GlcNac}_2$ to smaller species. In view of this data, it is my opinion that one of ordinary skill in the art, following the teachings of the application, could readily produce the pharmaceutical compositions recited in the claims.

16. All statements are made herein of my knowledge are true, and all statements made on information and belief are believed to be true. Further, these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent application issuing thereon.



Timothy Edmunds
Dated: 7/18/06

Exhibit A

Timothy Edmunds, Ph.D.
Vice President
Therapeutic Protein Research

Genzyme Corporation**PROFESSIONAL EXPERIENCE**

October 1989-Present **GENZYME CORPORATION**
Framingham MA

Research **2002-Present Vice President Therapeutic Protein**

1999-2002	Senior Scientific Director
1995-1999	Scientific Director
1992-1995	Associate Scientific Director
1990-1992	Senior Scientist
1989-1990	Staff Scientist II

Responsibilities

Responsible for the development and implementation of analytical strategies and structure/function studies to support research, preclinical and clinical development and regulatory approval (US, European and Japanese) for products from natural (human and plant), recombinant (bacterial, yeast and mammalian cell culture) and transgenic (goat and bovine) expression systems.

April 1986-October 1989 **DAMON BIOTECH,**
Needham Heights MA

1988-1989 **Director of Protein Chemistry.**
1986-1989 **Senior Scientist**

Responsibilities

Established the Protein Chemistry Department at Damon Biotech. Responsibilities included development of all purification and analytical procedures (enzymatic and ELISA assay development, protein sequencing and peptide mapping) for recombinant proteins (tPA, Urokinase and Monoclonal Antibodies). Provide purified proteins for pre-clinical studies by outside collaborators and US and Japanese partners.

EDUCATION

University of Newcastle upon Tyne

Newcastle upon Tyne, England

Ph.D, July 1983

Thesis Title: *Studies on Proteases from Muscle and Blood*

University of Liverpool

Liverpool, England

B.Sc, July 1978

ACADEMIC TRAINING

1985-1986 Harvard Medical School

Boston, Massachusetts

Postdoctoral Fellow in the Department of Physiology and Biophysics

1983-1985 University of Arizona,

Tucson Arizona

Postdoctoral Associate in the Muscle Biology Group

PATENTS

T. Edmunds and S. Foley, US Patent 4,920,051
Recovery of Urokinase Compounds. April 1990

T. Edmunds and S. Foley, US Patent 4,929,560
Recovery of Tissue Plasminogen Activator. May 1990.

INVITED PRESENTATIONS

3rd Annual Conference on Post-Translational Modifications, Washington D.C. Improving Therapies for Lysosomal Storage Diseases. November 2004.

Sixth European Working Group on Gaucher Disease. Barcelona Spain. "Cysteine Residues and α -glucocerebrosidase Stability: Implications for Improving Enzyme Replacement Therapy" October 2004

Glycomics Carbohydrates in Drug Development, Cambridge Massachusetts. "N-linked Glycosylation Friend or Foe." May 2003.

"The Impact of Post-Translational and Chemical Modifications on Protein Therapeutics." San Diego, Biochemical Comparison of Fabrazyme and Replagal. May 2002

XIIth Methods in Protein Sequence Analysis, Halkidiki, Greece. "Heterogeneity in therapeutic protein products: Analysis of post-translational modifications." September 1998.

24th Annual FACSS Conference, Providence, RI "Well-Characterized Biologicals, the Role of Mass Spectrometry in Recombinant Protein Analysis". October 1997

Specified Biologicals, Washington D.C. "Characterization Strategy for Antithrombin III: A Well Characterized Transgenic Protein". October 1997

Society for Glycobiology -Carbohydrate Mass Spectrometry Satellite Meeting, Boston, MA

"Mass Spectrometric analysis of transgenic glycoproteins". November 1996

Characterization of Biotechnology Pharmaceutical Products, Washington D.C.
"Cerezyme a Case Study" December 1995.

Royal Society of Chemistry Carbohydrate Group, University of Exeter, England. "Use of electrospray mass spectrometry for the rapid identification of glycosylation site heterogeneity" April 1994.

PUBLICATIONS

Zhou Q., Park S-H., Boucher S., Higgins E., Lee K. and Edmunds T. (2004) N-linked Oligosaccharide Analysis of Glycoprotein Bands from Isoelectricfocusing Gels. *Anal Biochem* 335 10-16

Lee, K., Jin, X., Zhang, K., Copertino, L., Andrews, L., Baker-Malcolm, J., Geagan, L., Qiu, H., Seiger, K., Barngrover, D., McPherson, J. M., and Edmunds, T. (2003). "A biochemical and pharmacological comparison of enzyme replacement therapies for the glycolipid storage disorder Fabry disease." *Glycobiology*, 13(4), 305-13.

Qiu, H., Edmunds, T., Baker-Malcolm, J., Kary, K. P., Estes, S., Schwarz, C., Hughes, H., and Van Patten, S. M. (2003). "Activation of human acid sphingomyelinase through modification or deletion of C-terminal cysteine." *J Biol Chem*, 278(35), 32744-52.

Roeber, D., Achari, A., Manavalan, P., Edmunds, T., and Scott, D. L. (2003). "Crystallization and preliminary X-ray analysis of recombinant human acid beta-glucocerebrosidase, a treatment for Gaucher's disease." *Acta Crystallogr D Biol Crystallogr*, 59(Pt 2), 343-4.

Zhou, Q., Kyazike, J., Edmunds, T., and Higgins, E. (2002). "Mannose 6-phosphate quantitation in glycoproteins using high-pH anion-exchange chromatography with pulsed amperometric detection." *Anal Biochem*, 306(2), 163-70

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Van Patten, S. M., Hanson, E., Bernasconi, R., Zhang, K., Manavalan, P., Cole, E. S., McPherson, J. M., and Edmunds, T. (1999). "Oxidation of methionine residues in antithrombin. Effects on biological activity and heparin binding." *J Biol Chem*, 274(15), 10268-76.

Edmunds, T. (1998) Cerezyme®: A case study. *Dev Biol Stand*, 96, 131-40

Edmunds, T., Van Patten, S.M., Pollock, J., Hanson, E., Bernasconi, R., Higgins, E., Manavalan, P., Ziomek, C., Meade, H., McPherson, J.M. and Cole, E.S. (1998). Transgenically produced human antithrombin: Structural and functional comparison to Human plasma-derived antithrombin. *Blood*, 91 (12), 4561-4571.

Lee, K. L., Albee, K. L., Bernasconi, R. J. and Edmunds, T. (1997). "The complete amino acid sequence of Ananain and a comparison to stem bromelain and other plant cysteine proteinases. *Biochem J*. 237 199-202.

Skrabut, E. M., Hebda, P. A., Samuels, J. A., Richards, S. M., Edmunds, T., Cunneen, M. F., Vaccaro, C. A., & McPherson, J. M. (1996). Removal of necrotic tissue with an amaranth-based enzyme-debriding preparation. *Wound Repair and Regeneration*, 4(4), 433-443.

Garone, L., Edmunds, T., Hanson, E., Bernasconi, R., Huntington, J. A., Meagher, J. L., Pan, B., & Gettins, P. G. (1996). Antithrombin-heparin affinity reduced by fucosylation of carbohydrate at asparagine 155. *Biochemistry*, 35(27), 8881-8889.

Cole, E. S., Lee, K., Lauziere, K., Kelton, C., Chappel, S., Weintraub, B., Ferrara, D., Peterson, P., Bernasconi, R., Edmunds, T., & et. a. (1993). Recombinant human thyroid stimulating hormone: development of a biotechnology product for detection of metastatic lesions of thyroid carcinoma. *Biotechnology*, 11(9), 1014-1024.

Cole, E. S., Nichols, E. H., Lauziere, K., Edmunds, T., & McPherson, J. M. (1991). Characterization of the microheterogeneity of recombinant primate prolactin: implications for posttranslational modifications of the hormone in vivo. *Endocrinology*, 129(5), 2639-2646.

Denman, J., Hayes, M., O'Day, C., Edmunds, T., Bartlett, C., Hirani, S., Ebert, K. M., Gordon, K., & McPherson, J. M. (1991). Transgenic expression of a variant of human tissue-type plasminogen activator in goat milk: purification and characterization of the recombinant enzyme. *Biotechnology*, 9(9), 839-843.

Edmunds, T., Nagainis, P. A., Satho, S. K., Thompson, V. F., & Goll, D. E. (1991). Comparison of the autolyzed and unautolyzed forms of mu- and m-calpain from bovine skeletal muscle. *Biochim Biophys Acta*, 1077(2), 197-208.

Wolfe, F. H., Sathe, S. K., Goll, D. E., Kleese, W. C., Edmunds, T., & Duperré, S. M. (1989). Chicken skeletal muscle has three Ca^{2+} -dependent proteinases. *Biochim Biophys Acta*, 998(3), 236-250.

Kleese, W. C., Goll, D. E., Edmunds, T., & Shannon, J. D. (1987). Immunofluorescent localization of the Ca^{2+} -dependent proteinase and its inhibitor in tissues of *Crotalus atrox*. *J Exp Zool*, 241(3), 277-289.

Edmunds, T., & Goldberg, A. L. (1986). Role of ATP hydrolysis in the degradation of proteins by protease La from *Escherichia coli*. *J Cell Biochem*, 32(3), 187-191.

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Goll, D. E., Edmunds, T., Kleese, W. C., Sathe, S. K., & Shannon, J. D. (1985). Some properties of the Ca^{2+} -dependent proteinase. *Prog Clin Biol Res*, 180, 151-164.

Edmunds, T., & Pennington, R. J. (1985). A high-molecular weight peptide hydrolase from rat skeletal muscle. *Prog Clin Biol Res*, 180, 235-237.

Goll, D. E., Shannon, J. D., Edmunds, T., Sathe, S. K., Kleese, W. C. and Nagainis, P. A. (1983) Properties and regulation of the Ca^{2+} -dependent proteinase. In "Calcium-Binding Proteins in Health and Disease" (deBernard, B., Scottocasse, G. L. Carafoli, E., Taylor, A. N., Vannaman, T.C. and Williams, R. J. P., ed) Elsevier Science Publishers, B. V., Amsterdam. pp. 19-35.

Edmunds, T., & Pennington, R. J. (1982). A high molecular weight peptide hydrolase in erythrocytes. *Int J Biochem*, 14(8), 701-703.

Edmunds, T., & Pennington, R. J. (1981). Mast cell origin of 'myofibrillar protease' of rat skeletal and heart muscle. *Biochim Biophys Acta*, 661(1), 28-31.

Exhibit B (page 1 of 3)

CHO cell expression in the presence of carbohydrate processing inhibitors.

Chinese Hamster Ovary (CHO) cells expressing glucocerebrosidase were thawed into growth medium containing DCS. Cells were expanded to 5 x 500ml spinners with Cytopore 2 microcarriers. On days 3, 5 and 7 each spinner was refed with ~80% volume exchange of fresh growth media. On Day 8, cultures were transitioned to serum-free 925 media +0.5mM DTT, pH 6.7. The first four exchanges in the serum free medium were discarded. The fifth serum-free media addition contained 0.5mM DTT and an inhibitor of carbohydrate processing that acts to inhibit the conversion of $\text{Glc}_1\text{Man}_2\text{GlcNac}_2$ to smaller species. Media was harvested after 48 hours and the spinners were refed with the same media. A second harvest followed 48 hours later and the harvests were pooled. Glucocerebrosidase was purified using standard techniques. The inhibitors were used at standard concentrations.

Analysis of GCR

The presence of oligomannose oligosaccharides was demonstrated by a mobility change on SDS PAGE following removal of oligomannose chains with Endoglycosidase H. and analysis of released oligosaccharides by mass spectrometry.

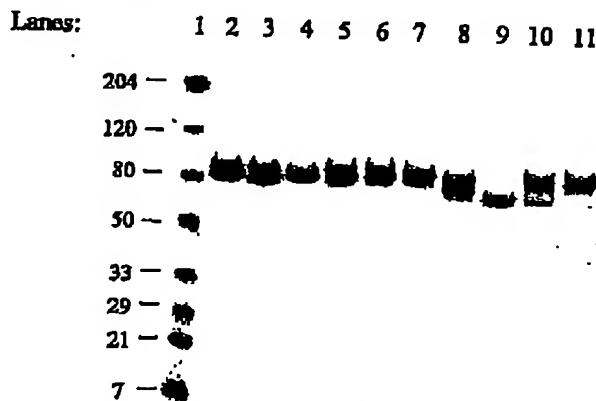
Prior to analysis, SP eluate pools were buffer-exchanged into 50 mM sodium citrate pH 5.7 to remove ethylene glycol and protein concentration determined by absorbance at 280nm. Aliquots of the protein were treated with Endo H (Roche) overnight in 50 mM sodium phosphate (pH 7.0) or 50 mM sodium citrate (pH 5.7). Samples of GCR (2.5 ug) with or without Endo H digestion were analyzed using a 4-12% SDS PAGE gel and stained using Coomassie blue.

Exhibit B (page 2 of 3)

Figure 1

4-12% SDS PAGE of purified glucocerebrosidase treated with or without Endo H:
Coomassie blue staining

MW
(Kd)



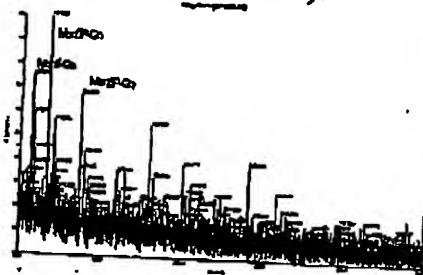
Lane 1, protein molecular weight standard. Lanes 2~6, glucocerebrosidase without Endo H pretreatment; lanes 7~11, glucocerebrosidase with Endo H pretreatment. Lanes 2 and 7, glucocerebrosidase in absence of inhibitor; lanes 3 and 8, glucocerebrosidase in the presence of deoxymannojirimycin; lanes 4 and 9, glucocerebrosidase in the presence of swainsonine; lanes 5 and 10, glucocerebrosidase in the presence of castanospermine; lanes 6 and 11, glucocerebrosidase in the presence of deoxynojirimycin.

Exhibit B (page 3 of 3)

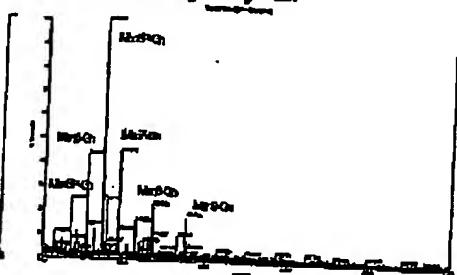
Glycans released by Endo H were dialyzed against 500 mL dH₂O and analyzed using a Voyager-DE PRO Biospectrometry Workstation. MALDI-TOF MS spectra were acquired in the positive-ion reflectron mode using sDHB as matrix.

Figure 2
MALDI-TOF MS analysis of Endo H released N-glycans from glucocerebrosidase:

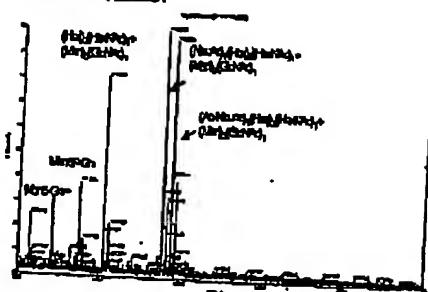
Control (without inhibitor):



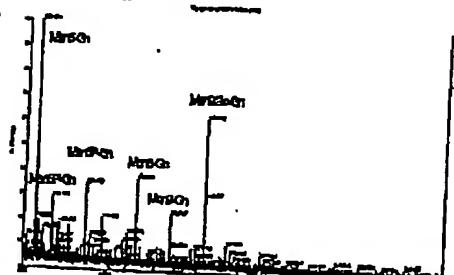
Deoxymannojirimycin:



Swainsonine:



Castanospermine:



Deoxymannojirimycin:

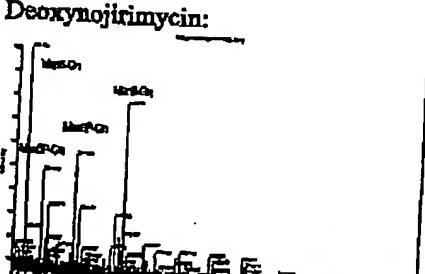


Exhibit C (page 1 of 2)
Human cell line expression in the presence of carbohydrate processing inhibitors.

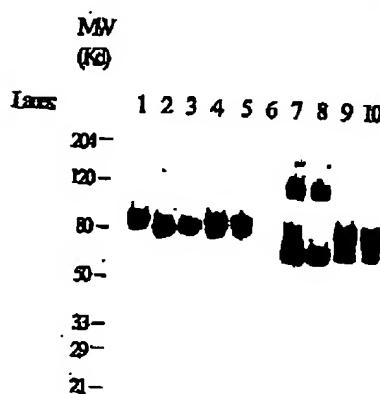
HeLa Cells

HeLa cells were plated into fifteen 15 cm dishes at 6E6 cells/dish in DMEM/10%FBS/1%Penn/Strep/1%L-Glutamine. 24 hours later, the cells were infected with Ad2/CMVGCR Lot #R2301B at multiplicity of infection = 250 in 925 media/10% FBS/1% Penn/Strep. 24 hours post infection, cells were washed with PBS and the inhibitors were added at the following concentrations in 925 media without serum/+ 1 mM DTT. Three dishes of infected HeLa were used per condition (3.18E7 cells for each condition). The inhibitors were used at standard concentrations.

24 hours following the addition of inhibitors, the cells and media were harvested.

Figure 1

Western Blot Analysis of glucocerebrosidase



The presence of oligomannose oligosaccharides was demonstrated by a mobility change on SDS PAGE following removal of oligomannose chains with Endoglycosidase H. Cell culture media containing 5 ng of glucocerebrosidase was treated with Endo H overnight and applied to a 4-12% SDS-PAGE gel and transferred to a PVDF membrane. glucocerebrosidase was identified using a biotinylated rabbit anti human glucocerebrosidase antibody.

Lanes 1~5, glucocerebrosidase without Endo H pretreatment; lanes 1~6, glucocerebrosidase with Endo H pretreatment. Lanes 1 and 6, glucocerebrosidase in absence of inhibitor; lanes 2 and 7, glucocerebrosidase in the presence of deoxymannojirimycin; lanes 3 and 8, glucocerebrosidase in the presence of swainsomine; lanes 4 and 9, glucocerebrosidase in the presence of castanospermine; lanes 5 and 10, glucocerebrosidase in the presence of deoxymannojirimycin.

Exhibit C (page 2 of 2)

HEK293 Cells

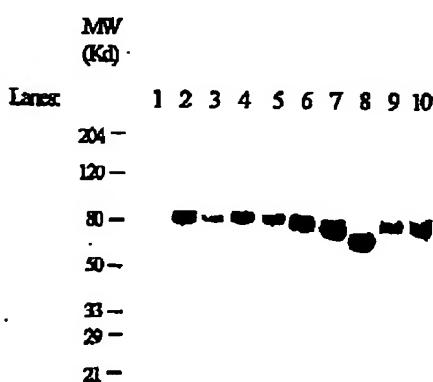
HEK293 cells containing Epstein-Barr virus nuclear protein (ATCC) were seeded at 9×10^6 cells/T150cm² flask with 25ml of DMEM w/ high glucose/4mM L-glutamine/10% Fetal Bovine Serum medium at 37 C in 6% CO₂ humidified incubator. The cells were transfected with pcDNA6 containing a human glucocerebrosidase sequence (under control of DNA backbone pcDNA6/VS/His-C, Invitrogen) using calcium phosphate precipitation method in DMEM w/ high glucose/4mM L-glutamine/1% Fetal Bovine Serum. At 4-6 hours post-transfection/incubation at 37 C in 2.5% CO₂ humidified incubator, media was replaced with 925 pH6.8-7.02 medium containing 50µM Dithiothreitol, +/- inhibitors at standard concentrations. After 72hour incubation at 37 C in 6% CO₂ humidified incubator the media was harvested and filtered.

Western Blot Analysis of glucocerebrosidase

The presence of oligomannose oligosaccharides was demonstrated by a mobility change on SDS PAGE following removal of oligomannose chains with Endoglycosidase H. Cell culture media containing 5 ng of glucocerebrosidase was treated with Endo H overnight and applied to a 4-12% SDS-PAGE gel and transferred to a PVDF membrane. glucocerebrosidase was identified using a biotinylated rabbit anti human glucocerebrosidase antibody.

Figure 2

Western blot of glucocerebrosidase treated with or without Endo H:



Lanes 1~5, glucocerebrosidase without Endo H pretreatment; lanes 1~6, glucocerebrosidase with Endo H pretreatment; Lanes 1 and 6, glucocerebrosidase in absence of inhibitor; lane 2 and 7, glucocerebrosidase in the presence of deoxymannojirimycin; lanes 3 and 8, glucocerebrosidase in the presence of swainsonine; lanes 4 and 9, glucocerebrosidase in the presence of castanospermine; lane 5 and 10, glucocerebrosidase in the presence of deoxymannojirimycin.